A MICROELECTROPHORETIC AND MICROIONOPHORETIC TECHNIQUE*

by

(26)
21 pp

E. L. Durrum, Capt., M.C.

from

Medical Department Field Research Laboratory
Fort Knox, Kentucky
15 March 1949

*Sub-project under Studies of Body Reactions and Requirements under Varied Environmental and Climatic Conditions. Approved 31 May 1946.
M.D.F.R.L. Project No. 6-64-12-06-(18).

Project No. 6-64-12-06
Sub-project MDFRL 06-(18)
MEDEA

15 March 1949

ABSTRACT

A MICROELECTROPHORETIC AND MICROIONOPHORETIC TECHNIQUE

OBJECT

The object of this investigation was to develop a simplified, rapid method for the separation of mixtures of amino acids, peptides and proteins as they occur in biological products, that would be applicable to separations of the minute amounts of material such as may be available from small laboratory animals.

RESULTS

This objective has been achieved by the development of a microelectrophoretic and microionophoretic technique in which an electrical potential is applied across the ends of strips of filter paper saturated with buffer or other electrolyte solutions. To these strips are applied, at narrowly circumscribed intermediate areas, mixtures of amino acids, peptides, and/or mixtures of proteins to be separated. The positions to which components have migrated are determined in the case of amino acids by spraying the dried strips with ninhydrin and in the case of proteins, by coagulating the protein *in situ* on the paper strips and then treating the paper strip with a dye selective for the coagulated protein constituents but easily washed from the filter paper in zones free of protein. Radioactive constituents have been located by autoradiography of the strips.

This method has proved suitable for the separation of complex amino acid mixtures. For example, in a single step, lysine, arginine, histidine, glutamic and aspartic acids have been separated from mixtures containing 14 other monoamino-monocarboxylic acids. Blood serum has been fractionated into albumen and other fractions. Radioactive inorganic iodide ion has been separated from radioactive iodine bound by protein in thyroid proteins.

CONCLUSIONS:

The method described promises to have wide applicability. It has the advantage of being rapid. It requires only very simple inexpensive apparatus and relatively unskilled personnel. It seems possible that eventually, the method as applied to proteins, for example, is capable of yielding part of the information heretofore available only with the elaborate Tiselius apparatus.

RECOMMENDATIONS

It is recommended that the investigation be continued in an effort to develop the quantitative aspects of the process and to explore its clinical applications.

Submitted by:
E. L. Durrum, Capt., M.C.

Approved: Ray G. Daggs
RAY G. DAGGS
Director of Research

Approved: F. J. Knoblauch
FREDERICK J. KNOBLAUCH
Lt. Col., M.C.
Commanding

A MICROELECTROPHORETIC AND MICROIONOPHORETIC TECHNIQUE

I. INTRODUCTION

In performing electrophoretic and ionophoretic separations, several investigators have utilized an electrical potential applied across various packing materials intended to stabilize migrating boundaries by preventing convection currents in the electrolytes employed. Strain (1) combined ionophoresis with chromatographic adsorption in the conventional Tswett absorption column and mentioned utilizing columns filled with cotton for this purpose. Coolidge (2) was able to separate protein constituents in a column packed with ground glass wool across which a potential was applied. Consden, Gordon and Martin (3) described an ionophoretic technique suitable for the separation of certain amino acids which was carried out in silica jelly slabs made up with various buffers. These investigators utilized paper pulp to reinforce the mechanical strength of the silica jelly slabs employed. They also reported an experiment in which they filled their trough with "paper powder saturated with liquids to be analyzed" but abandoned this method because current densities optimum for their purpose could not be employed. Butler and Stephen (4) have utilized asbestos fiber packed in a segmented polystyrene plastic tube and reported separating glycine from glycylglycine at pH 9.3 in this apparatus. None of the above processes was adapted to the separation of small quantities of material.

Recently, Haugaard and Kroner (5) applied electrical potentials across paper partition chromatographs during their development with phenol. They wove thin, flat, metallic electrodes into the edges of the paper which had first been treated with phosphate buffer solution and then dried prior to development with phenol. They reported that the degree of separation of basic and acidic amino acids attainable by paper partition chromatography was enhanced by this expedient. Though their process is applicable to the separation of minute quantities of amino acids, it does not appear to be applicable to protein separstions.

This report is concerned with a microionophoretic or microelectrophoretic technique which has been found useful for the separation of both amino acids and protein constituents in which an electrical potential is applied across the ends of strips of filter paper saturated with buffer or other electrolyte solutions to which are applied, at narrowly circumscribed intermediate areas, mixtures of amino acids, peptides or proteins to be separated. The positions to which components have migrated are determined in the case of amino acids and peptides by spraying the dried strip with ninhydrin (Consden, Gordon and Martin (6)), and in the case of proteins, by "fixing" the protein *in situ* on the paper strips by heat or by coagulation with chemical agents followed by treating the paper strip with a dye selective for the coagulated protein constituents but easily washed from the filter paper in zones free of protein. A third method which has been employed either alone or in combination with the above methods in cases where radioactive constituents are concerned is that of making autoradiographs of the dried or "fixed" strips. The practical applicability of this method appears to be wide enough to make it desirable to report at this time, although its

theoretical aspects remain to be investigated more thoroughly.

II. EXPERIMENTAL

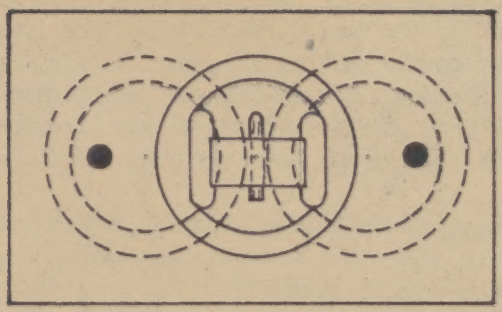
In preliminary experiments, narrow (1 cm.) strips of filter paper (about 0.16 mm. thick) were saturated with buffer solutions and the strips draped between two vessels containing the buffer solutions into which were inserted carbon rod electrodes. About the middle of the strips, a drop of serum or amino acid mixture was applied and then a potential of a few hundred volts applied across the carbon rods. These experiments served to show that separations could be practically effected in reasonable periods of time. There were, however, two disadvantages: (1) ill-defined zones of amino acids or proteins were obtained because of the syphoning of the buffer solutions to the low point of the paper with consequent "flooding" in this area; and (2) evaporation from the surface of the paper and temperature could not be controlled readily.

These difficulties were partially avoided by employing a glass bridge arrangement with a filter paper strip placed between somewhat wider plate glass strips resting on the electrode vessels. However, during many of the experiments, puddles of electrolyte were observed to collect irregularly and lateral to the edges of the paper with attendant uncertainties as to uniformity of field strength and as to diffusion of the amino acids into these areas.

This puddling of electrolytes was believed to be due to capillary action between the glass plates in the areas lateral to the paper strip. It was observed that this effect could be minimized by superposing at least three strips of filter paper which then separated the plates at the edges by at least about 0.5 mm. This is a satisfactory method for multiple strips, but is not suitable for single strips. The apparatus was further modified, therefore, to permit the use of single thickness strips of filter paper to which the electrolyte could be confined in a reproducible manner.

The experiments on which the present report is based were carried out in the apparatus of the type illustrated in Figure 1. The apparatus is comprised of two 150 ml. glass tumblers carrying a lucite plate which seals their tops and supports an inverted L-shaped glass rod. The latter serves to support the apex of the filter paper strips which are draped over it. The ends of the strips pass into the electrolyte solution in the tumblers through slots in the lucite plate. Holes in the plate carry ordinary uncored arc carbon electrodes 8 mm. in diameter. The strips are isolated from the atmosphere by a third inverted 150 ml. tumbler. Annular grooves serve to improve the stability of this arrangement so that no external supports are required.

A larger version of the apparatus having electrode vessels of 500 ml. capacity and wide enough to support seven 1 cm. strips in parallel has proved to be quite convenient and useful when it is desired to compare known and unknown substances simultaneously under identical experimental conditions.



TOP VIEW

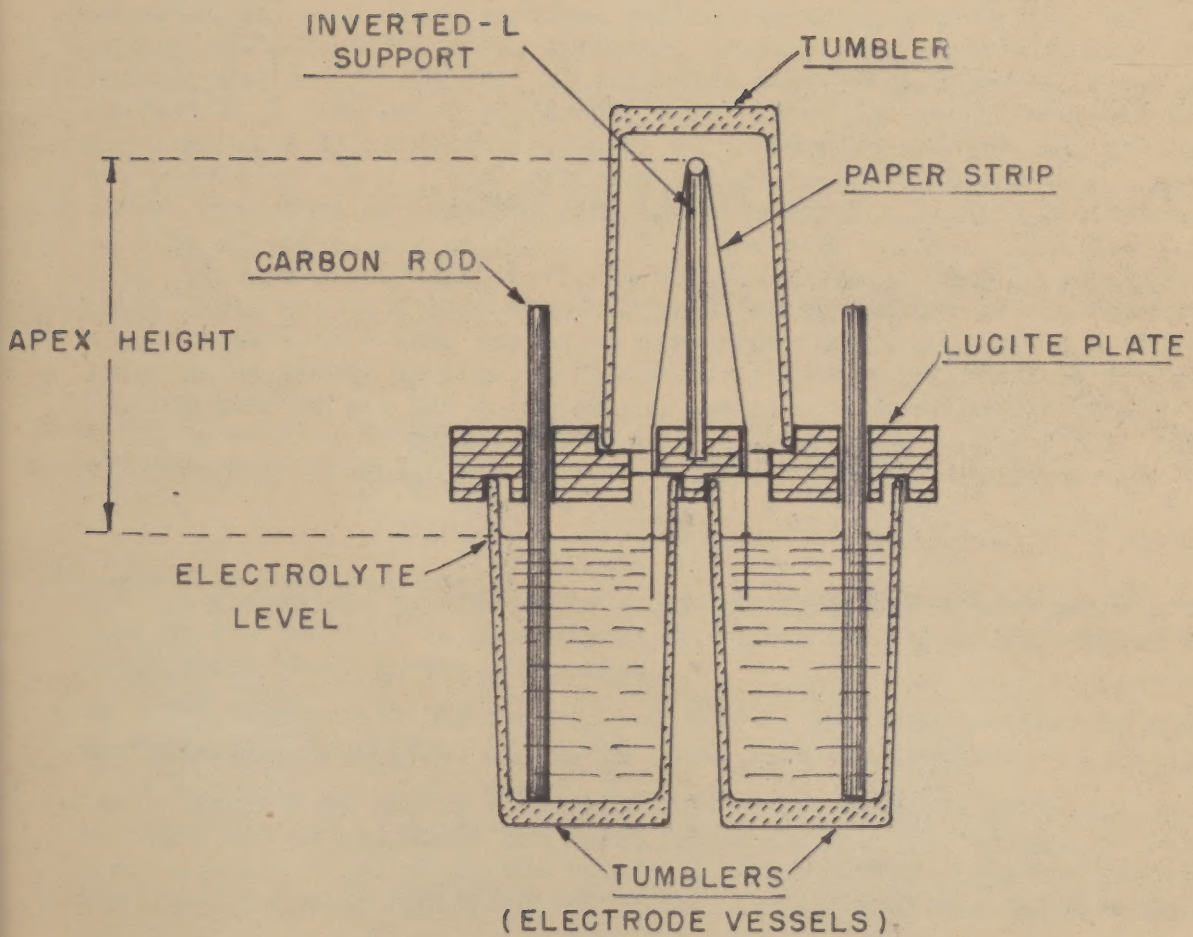


FIG. 1. DIAGRAM OF APPARATUS.

To adapt the apparatus shown in Figure 1 for experiments of long duration the tumbler electrode vessels were replaced with U-tubes in order to separate the electrode reaction zones from the paper strip ends by a greater distance. This modified apparatus consisted of two U-tubes with one limb of each constricted to a diameter slightly larger than the diameter of the carbon rod which it carried. The other two limbs of the U-tubes were brought out through a No. 10 rubber stopper. In a third hole, extending partly through the stopper, was inserted an inverted L-shaped glass rod which supported the apex of the filter paper strip. A short length of 2 inch (outside diameter) glass tube, with top closed by another No. 10 rubber stopper carrying a thermometer, isolated the system from the atmosphere. Practically for the separations described here, the apparatus shown in Figure 1 serves equally well and has the advantage of greater convenience.

In all of the experiments described, filter paper strips cut from 32 cm. circles of Whatman No. 2 paper were used. Except where otherwise indicated, strips 1 cm. wide were employed.* In cases where strips longer than 32 cm. are needed, shorter strips can be spliced with about 3 mm. overlap and platinum wire staples.

The use of this method is illustrated in the following experiments:

Experiment I - Separation into five fractions of an amino acid mixture** comprising 19 amino acids: arginine, lysine, histidine, glutamic acid, aspartic acid, glycine, alanine, valine, leucine, isoleucine, serine, threonine, cystine, methionine, tyrosine, tryptophane, phenylalanine, proline, hydroxyproline. Eighty ml. of potassium acid phthalate-sodium hydroxide buffer*** pH 5.9 (glass electrode) were placed in each electrode vessel of the apparatus illustrated in Figure 1. A pencil mark (x) was made across the middle of a 1 x 32 cm. strip of filter paper which was then draped across the glass support rod with the ends dipping about 1 cm. below the surface of the buffer solution in the electrode vessels. The apex of the paper strip was 14.5 cm. above the solution level. When in position, the paper strip was saturated with buffer solution applied to the apex with a medicine dropper. This served to wash from the paper strip any traces of amino acids picked up from the hands in the course of previous manipulations. The top tumbler was put in place and the apparatus allowed to stand for about 30 minutes to permit excess buffer to drain from the paper. About 20 micrograms of amino acid mixture in the form of a dry powder was then

---

* A convenient aid for cutting uniform strips is a centimeter grid fastened to the board of an ordinary paper cutter and for which a sheet of millimeter graph paper serves admirably.

** The amino acid mixture was prepared by grinding together in a mortar, equimolecular quantities of the 19 amino acids enumerated.

*** Prepared by mixing 50 ml. of 0.2M. potassium acid phthalate and 43 ml. of 0.2M. sodium hydroxide and diluting to 400 ml. (The initial pH was measured with the glass electrode and found to be 5.93. After the experiment was completed (120 min.), the pH of the anode vessel was found to be 5.91 and the cathode 6.01.

applied to the paper strip at the reference mark.

A potential of 600 volts direct current (supplied by a well filtered full wave rectifier) was applied across the carbon electrodes in series with a milliameter and rheostat. The current was maintained at 1.0 milliampere by frequent adjustment of the rheostat for a period of 120 minutes. Then the paper strip was transferred to a glass drying rack using forceps to avoid finger marks and taking care to maintain the apex upward during drying in order to prevent excess buffer at the ends of the paper from running back toward the apex and "smearing" the amino acid zones. The paper strip was dried in an oven at 90 C. for 5 minutes, then removed and sprayed with a 0.25% ninhydrin solution in water-saturated-butanol (Williams & Kirby (7)) and replaced in the oven for 5 minutes. The strip showed the following pattern (with all measurements to the center of the spot concerned): (a) toward the anode, 75 mm. from the reference mark (x) a bluish spot corresponding to aspartic acid; 60 mm. from the reference mark, a lavender spot corresponding to glutamic acid; (b) toward the cathode, 4 mm. from the reference mark a dense mauve spot corresponding to the monoamino-monocarboxylic acid group; 23 mm. from the reference mark a greyish spot corresponding to histidine* and at 43 mm. a lavender-rose spot corresponding to arginine and lysine which were not completely separated in this experiment. A photograph of the significant portion of this strip is shown in Figure 2.

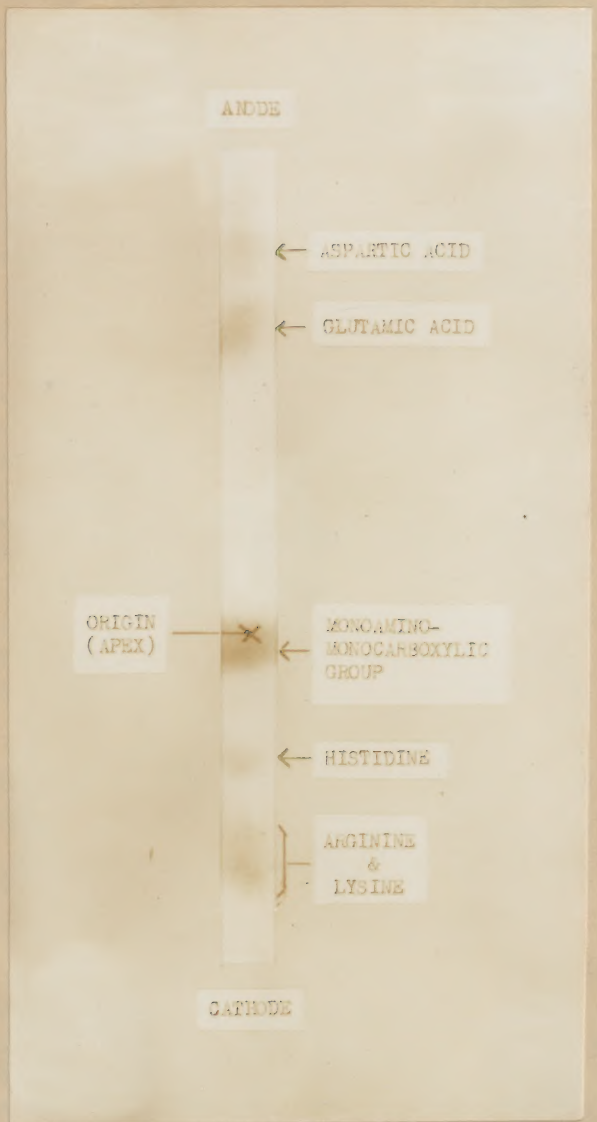
To separate a solution rather than dry crystals, the following variation in technique is employed: The paper strip to be used is draped on a glass drying rack after the reference pencil mark is made and washed down by directing several milliliters of distilled water at the apex. The strip is air dried and, handled with forceps, inserted into the apparatus as described above. Next, about 0.01 ml. of hydrolysate or protein solution, such as blood serum, is applied to the reference mark. Then, very carefully, buffer solution is applied with a medicine dropper <u>below</u> the apex of the strip at equal distances from the apex on either side, permitting the buffer to flow upward to the drop position (apex) by capillarity. In this manner, the solution is prevented from running down the filter paper as has been found to happen invariably if even a minute drop of solution is applied to pre-saturated though drained paper with a resultant lack of sharpness in the patterns obtained. When this variation is used, it is not necessary to wait more than about 10 minutes before applying the potential.

The above variation of technique is illustrated in the following example:

<u>Experiment II</u> - Separation of human serum: 0.01 ml. of serum was applied from a micro-pipette to the reference mark of a 1 cm. paper strip as described above. Immediately, a 0.05 molar sodium diethylbarbiturate buffer solution

---

* It has been observed that pH 5.9 gives a good separation of the histidine from the arginine-lysine zone and the monoamino-monocarboxylic zone. As the pH is increased, the histidine tends to migrate at a velocity closer to the latter group, merging with it at about pH 6.6. As the pH is decreased, the reverse has been observed with the histidine zone merging with the arginine-lysine zone at about pH 5.2.



Electrolyte:	Buffer (pH 5.9 - see text) Potassium Acid Phthalate- Sodium Hydroxide
Duration:	120 Minutes
Current:	1.0 ma./cm. (width) Initial 600 Volts Final - not recorded
Paper:	Whatman No. 2
Apex Height:	14.5 cm.

FIG. 2 SEPARATION OF 19 AMINO ACIDS INTO 5 GROUPS
(EXPERIMENT I)

(pH 8.6) was applied. A potential of about 350 volts was applied through a rheostat for 180 minutes. The current was maintained at 0.5 milliamperes by frequent adjustment of the rheostat. At the end of the run the strip was removed and dried for 5 minutes in an oven at 100°C.*, then immersed for 5 minutes in a saturated solution of mercuric chloride in 95% alcohol to which had been added 1 gm./100 ml. bromphenol blue (tetrabrom-phenolsulfonphthalein). The strip was then removed and washed for 10 minutes in running tap water. The strip was then dried. Four distinct blue zones were visible, all located toward the anodal side of the reference mark: the first, 35 mm. from the reference mark, corresponding to albumen; the second at 25 mm., probably corresponding to alpha$_1$ globulin; the third at 15 mm., corresponding to alpha$_2$ globulin and the fourth, 6 mm. from the reference mark, corresponding to beta globulin. A fifth zone was located 13 mm. toward the cathode corresponding to gamma globulin. The establishment of identity of these protein zones is discussed later in this report.

Experiment III - Separation of a mixture of alanine, valine, proline and tryptophane in the apparatus of larger dimensions. In this apparatus, 500 ml. of 5N. acetic acid was placed in each electrolyte vessel. The apex height in this experiment was 11.5 cm. above the fluid level. Five paper strips were supported, washed down and saturated with electrolyte as described above. On one strip, a few micrograms of a mixture of these amino acids was placed at the reference mark (x) and on each of the other strips, one of the amino acids of the mixture was placed at the reference mark. A potential of 580 volts was applied across the carbon electrodes. The initial current was 1.5 milliamperes per 5 cm. (width). After 120 minutes, the current had risen to 1.7 milliamperes per 5 cm. At this time, the strips were removed, dried and sprayed with ninhydrin. Portions of the resulting strips are shown in Figure 3.

Experiment IV - Separation of a mixture of glycine, isoleucine, phenylalanine and hydroxyproline. In an experiment exactly analogous to Experiment III, a mixture of the above amino acids was separated as illustrated in Figure 4. (The faint zone on the hydroxyproline strip represents accidental contamination with isoleucine.)

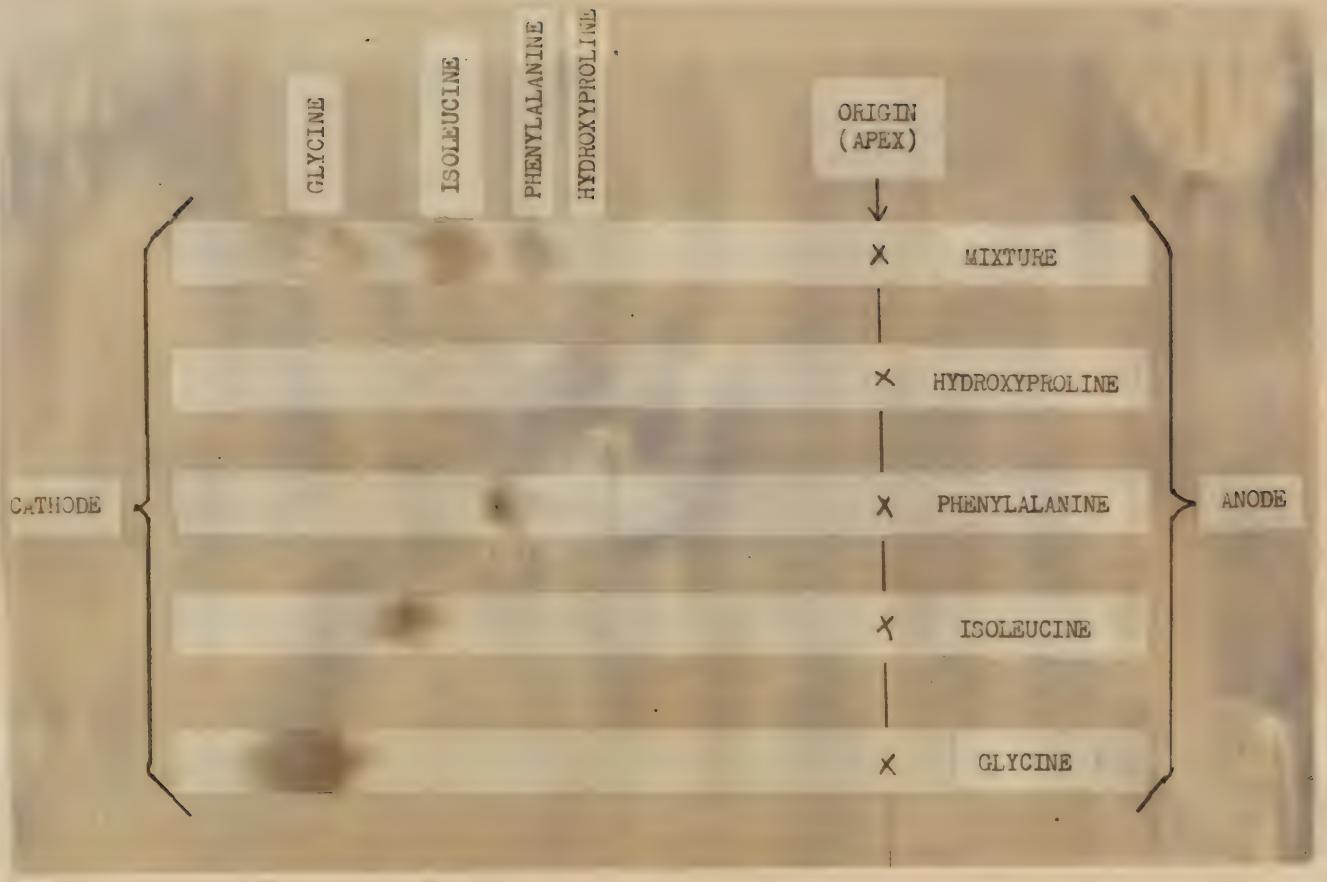
Experiment V - Separation of glycylglycine from glycyl-l-leucine in the apparatus of Experiments III and IV. Five strips were employed, to one of which a mixture of glycylglycine and glycyl-l-leucine was applied and to each of the remaining strips only one of these substances. Glycine and l-leucine were added to separate strips for comparison. The separation attained at the end of two hours is illustrated in Figure 5.

---

* This heating has been observed to result in the serum proteins being more deeply stained than when the strips are coagulated by mercuric chloride-ethyl alcohol-dye mixture alone. This is illustrated in Figure 9, the barbiturate separation strips having been coagulated without pre-oven drying compared to the phosphate buffer strips which were oven dried before treatment with the mercuric chloride-dye mixture.

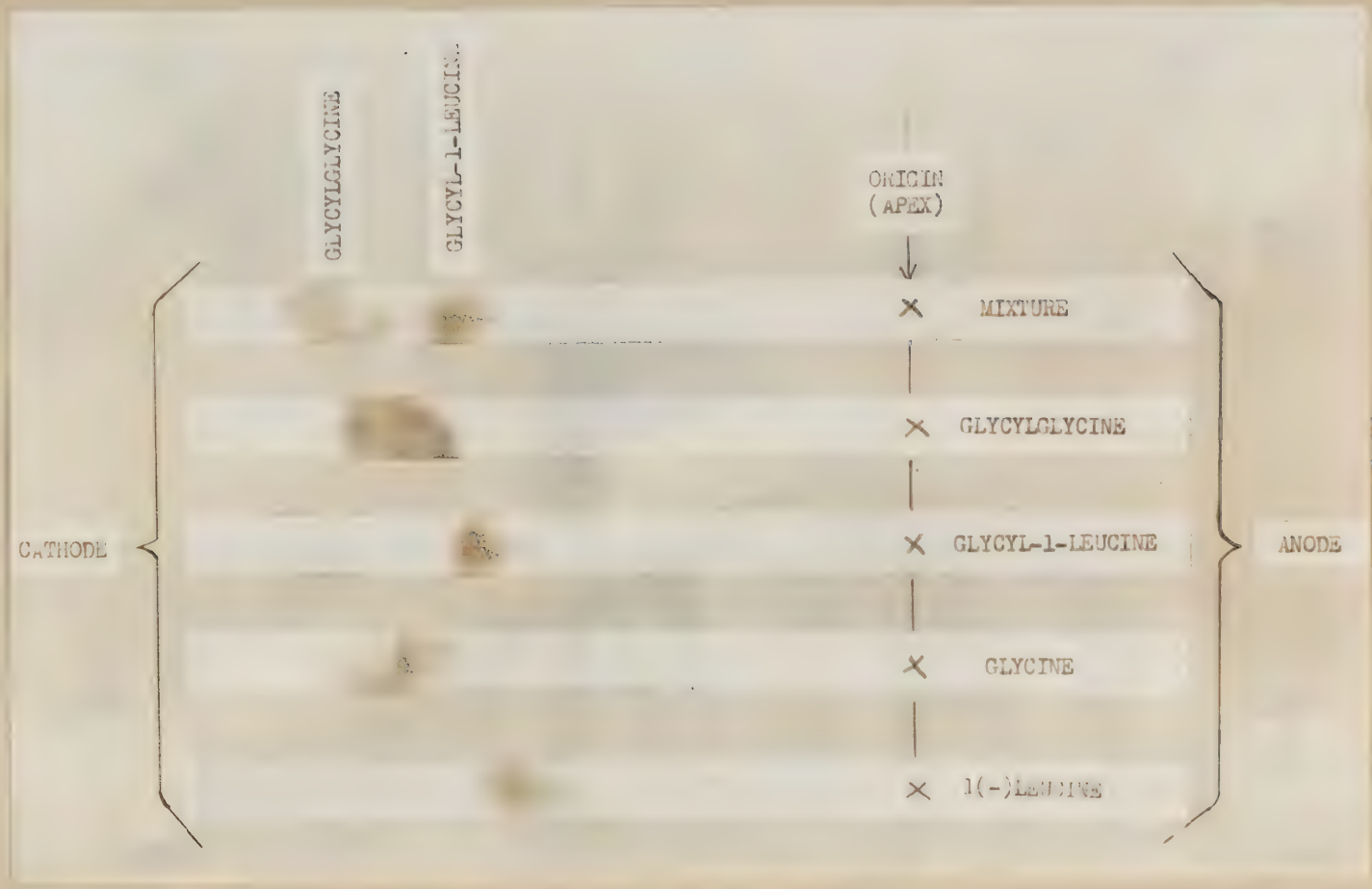
Electrolyte:	5N. Acetic Acid (pH 1.7)
Duration:	120 Minutes
Current:	Initial 1.5 ma./5 cm. (width)
	Final 1.7 ma./5 cm. (")
Potential:	580 Volts
Paper:	Whatman No. 2
	(5-1 cm. Strips in Parallel)
Apex Height:	11.5 cm.

FIG. 3 SEPARATION OF ALANINE, VALINE, PROLINE AND TRYPTOPHANE (EXPERIMENT III)



Electrolyte:	5N. Acetic Acid (pH 1.7)
Duration:	120 Minutes
Current:	Initial 1.5 ma./5 cm. (width)
	Final 1.7 ma./5 cm. (")
Potential:	580 Volts
Paper:	Whatman No. 2
	(5-1 cm. Strips in Parallel)
Apex Height:	11.5 cm.

FIG. 4 SEPARATION OF GLYCINE, ISOLEUCINE, PHENYLALANINE AND HYDROXYPROLINE (EXPERIMENT IV)



Electrolyte:	5N. Acetic Acid (pH 1.7)
Duration:	120 Minutes
Current:	Initial 1.5 ma./5 cm. (width)
	Final 1.7 ma./5 cm. (")
Potential:	580 Volts
Paper:	Whatman No. 2
	(5-1 cm. Strips in Parallel)
Apex Height:	11.5 cm.

FIG. 5 SEPARATION OF GLYCYLGLYCINE AND GLYCYL-l-LEUCINE (EXPERIMENT V)

Experiment VI - Reproducibility of parallel runs. The reproducibility of this method on parallel runs is illustrated by Figure 6 which shows sections of the paper strips obtained in a simultaneous run when a few micrograms of crystalline phenylalanine was applied to the origin of all strips. In this experiment, the electrolyte was 5N. acetic acid, the initial current 1.8 milliamperes per 6 cm. and the final current (120 minutes later) 1.7 milliamperes per 6 cm. (Ordinarily, the current has been observed to increase during the course of the runs. Rather marked line voltage fluctuations are sometimes noted which perhaps explain why the final current was recorded lower than the initial value.) The mean position of the phenylalanine was found to be 58.5 mm. from the origin with a standard deviation of ±3.27 mm. It is evident that the reproducibility of parallel runs is of sufficient degree ordinarily to permit selection of "matching pairs of acids" as, for example, is illustrated in Figures 3, 4, and 5.

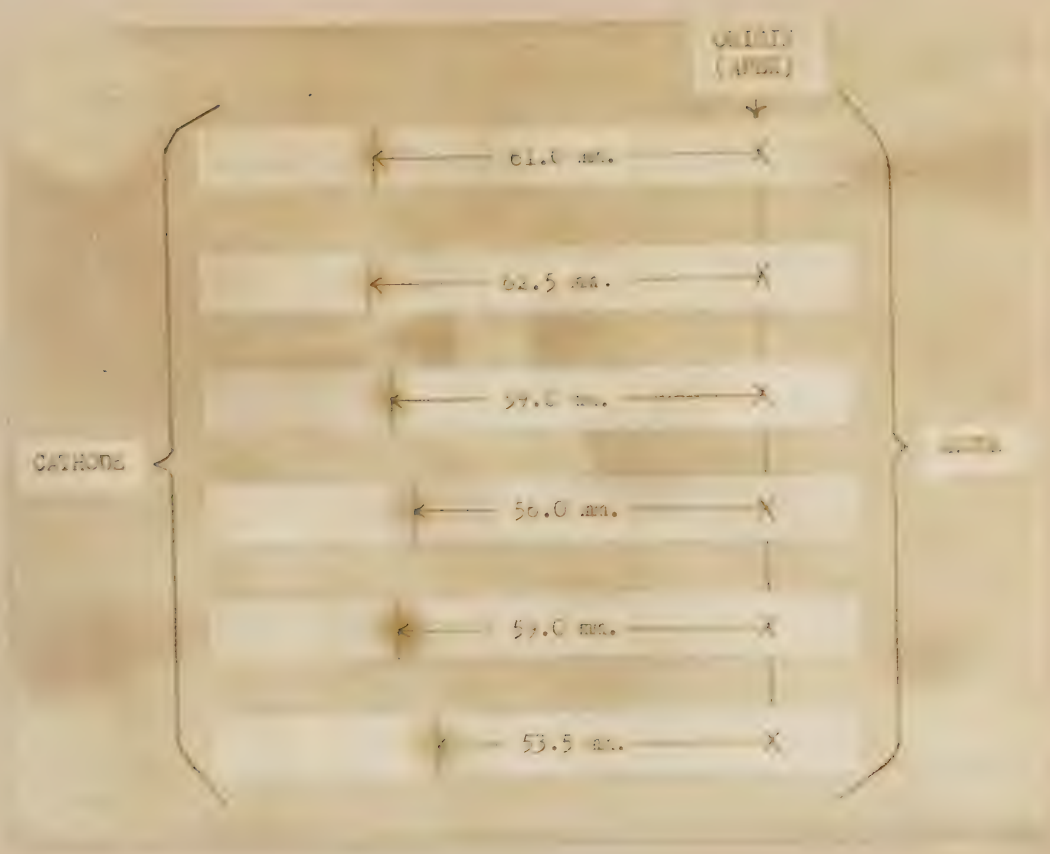
Experiment VII - Rate of migration of phenylalanine. Figure 7 illustrates the findings in an experiment in which the migration of phenylalanine toward the cathode was measured as a function of time. Six strips were set up in parallel with the apparatus previously described, the electrolyte being 5N. acetic acid. At 30 minute intervals strips were removed. During the course of the experiment under a potential of 580 volts, the current per strip averaged 0.3 milliamperes. It is evident that the migration of this amino acid down the paper under the conditions of these experiments is not a linear function of time.

Experiment VIII - Comparison of zones derived from separated electrophoretic components with whole serum. Figure 8 illustrates an experiment in which electrophoretic components separated in a Tiselius apparatus are compared with the whole serum pattern from which these components were derived. In the electrophoretic apparatus of Moore and White (8), a human serum sample was separated utilizing 0.1M. barbiturate buffer (pH 8.6, Longsworth (9)) into the following components:

a. Albumen (from Zone IV ascending limb of Tiselius cell).
b. A mixture of albumen plus alpha$_1$ globulin (from Zone V).
c. Pure gamma globulin (from Zone I descending limb).
d. A mixture of gamma globulin plus beta globulin (from Zone II).
e. A mixture of gamma, beta and alpha$_2$ globulins (from Zone III).

Paper patterns of these fractions were prepared under the similar conditions enumerated in Figure 8. The results of this experiment appear to establish the identity of all the components except alpha$_1$ globulin. It will be noted that in Figure 8, paper patterns IV and V do not differ appreciably although alpha$_1$ globulin is presumably present in pattern V only. The probable explanation is that since this component is present in such low concentration it is scarcely visible on the whole serum pattern; it is then not surprising that it is not more evident in pattern V which material was diluted in the course of the prior Tiselius separation.*

* This question could probably be answered by studying a pathological serum such as nephrotic serum which exhibits an increased alpha component.



Electrolyte:	5N. Acetic Acid (pH 1.7)
Duration:	120 Minutes
Current:	Initial 1.8 ma./6 cm.
	Final 1.7 ma./6 cm.(see text)
Potential:	580 Volts
Paper:	Whatman No. 2
	(6-1 cm. Strips in Parallel)
Apex Height	11.5 cm.

FIG. 6 REPRODUCIBILITY OF PARALLEL RUNS WITH PHENYLALANINE (EXPERIMENT VI)

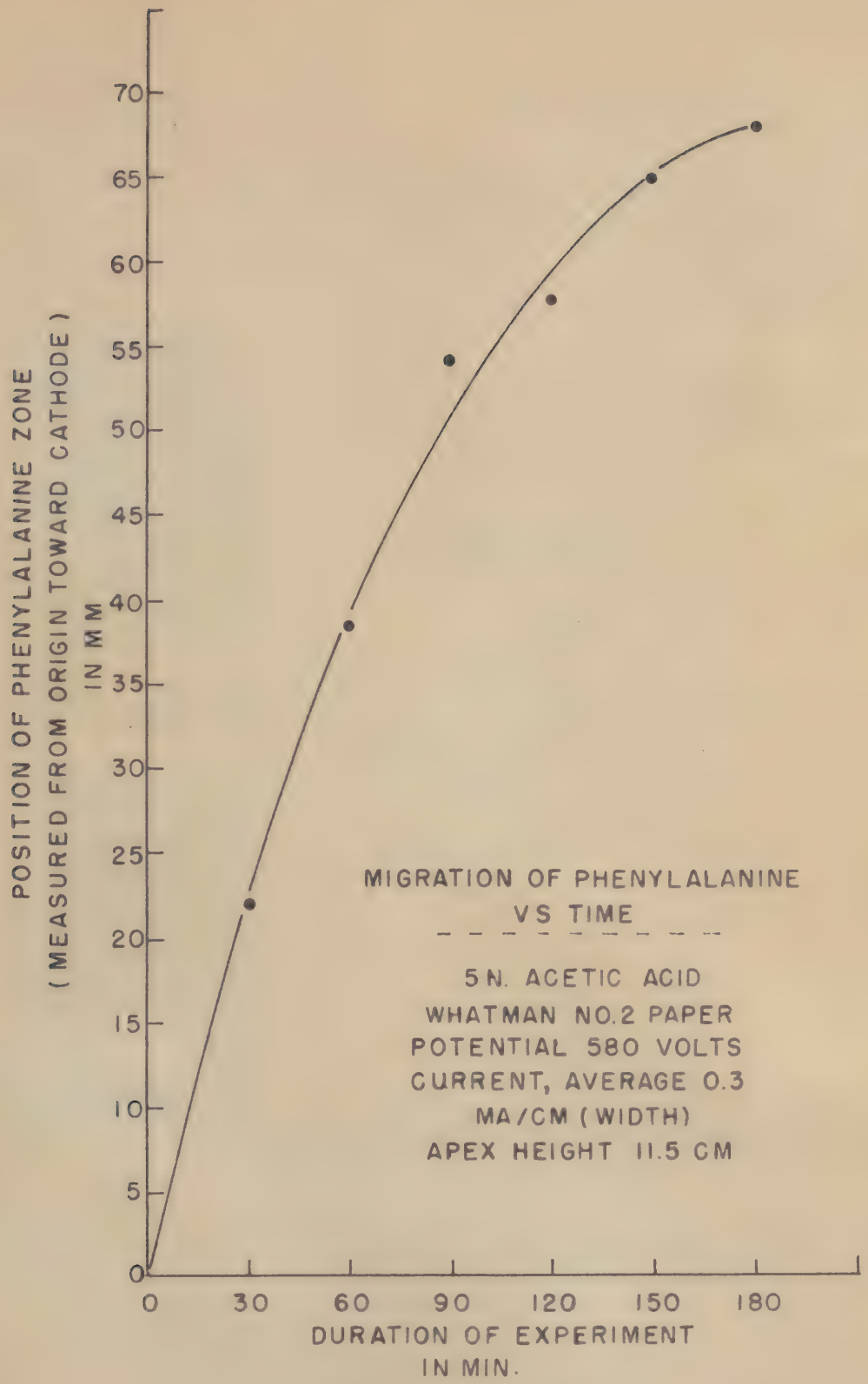
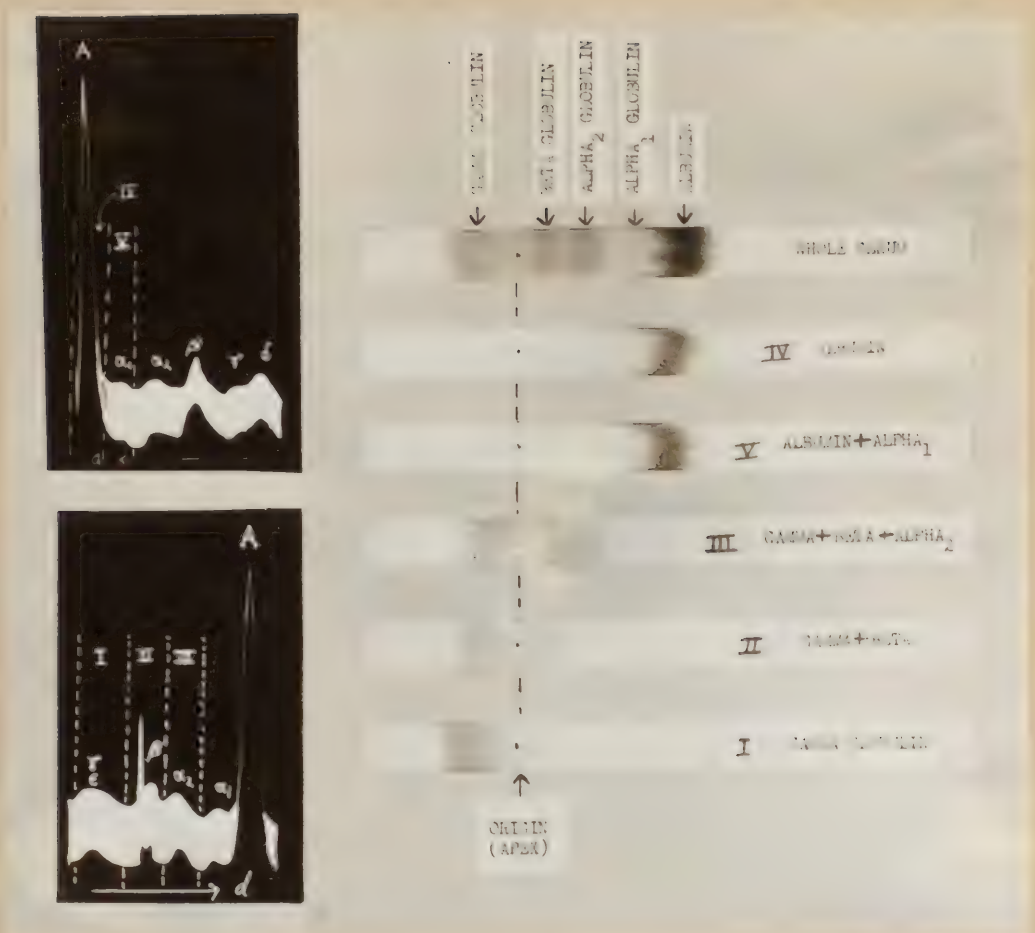


FIG. 7 MIGRATION OF PHENYLALANINE (EXP. VII)



Electrolyte:	Barbiturate Buffer (pH 8.6) 0.05M. Sodium Diethylbarbiturate 0.01M. Diethylbarbituric Acid
Duration:	180 Minutes
Current:	0.5 ma./cm. (width)
Potential:	Initial 300 Volts Final - not recorded
Paper:	Whatman No. 2
Apex Height:	13.5 cm.

FIG. 8 COMPARISON OF SEPARATED COMPONENTS WITH WHOLE SERUM (EXPERIMENT VIII)

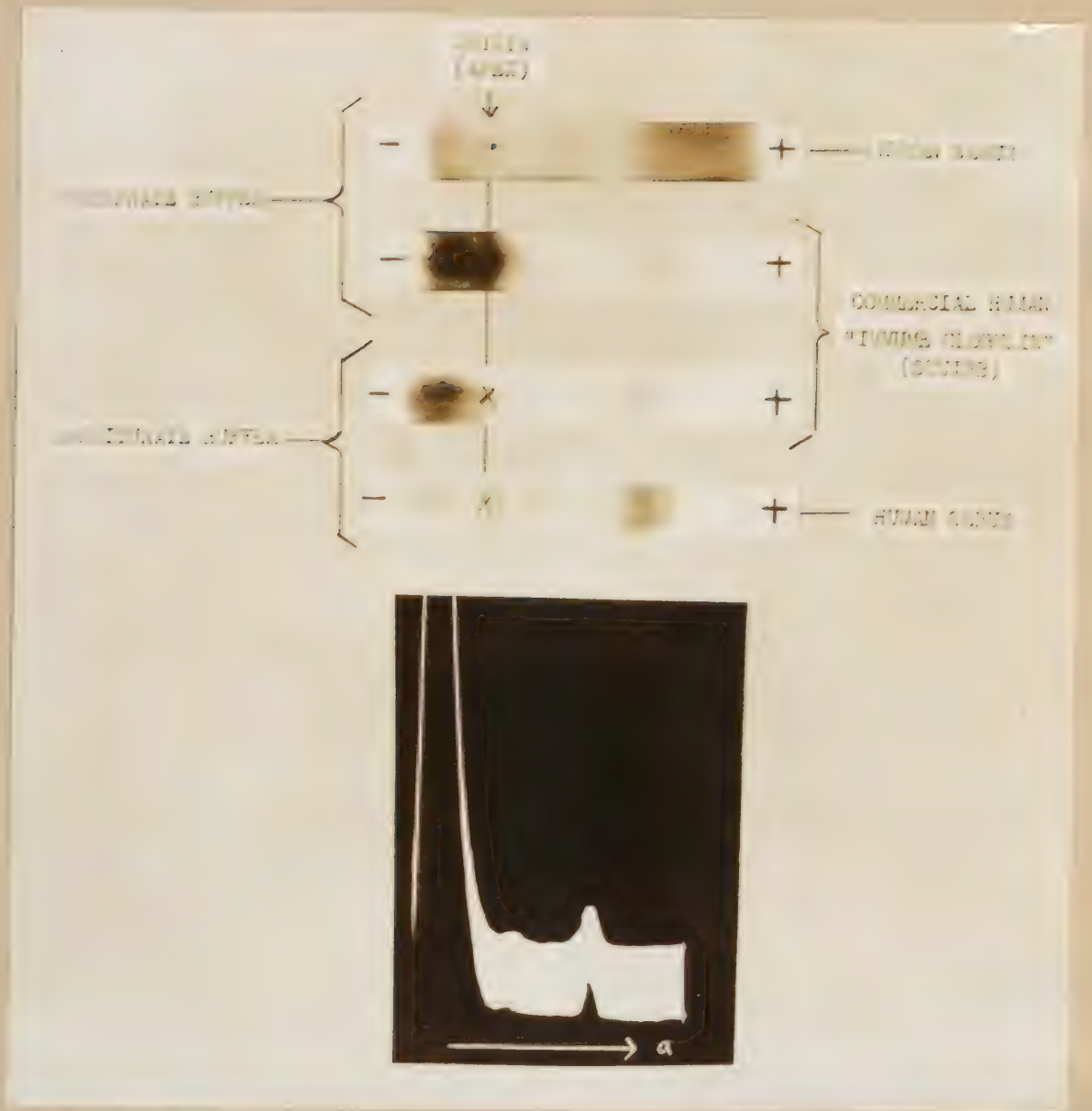
Experiment IX - Comparison of zones derived from "immune globulin" and reference serum with electrophoretic patterns. A similar analysis of a sample of commercial human "immune globulin" (Squibb) appears to be valid. Figure 9 illustrates an electrophoretic pattern prepared with 0.02 phosphate-0.15M. sodium chloride buffer (pH 7.4) in comparison with paper strips prepared with 0.05M. barbiturate buffer, pH 8.6, and 0.02M. phosphate buffer (without added sodium chloride), pH 7.6.

The component migrating most rapidly appears to be albumen from comparison with the serum patterns. Superior resulution and correspondence seems in this case to be found with the barbiturate buffer although a greater migration has occurred in shorter time with the phosphate buffer, undoubtedly due to the greater field strength in this experiment.

Experiment X - Comparison of human plasma and serum patterns. Figure 10 illustrates the patterns obtained in an experiment in which a serum and a heparin plasma derived from the same sample of human blood were separated on paper with barbiturate buffer. Control experiments have established that heparin does not stain with bromphenol blue under the conditions used in these experiments. Therefore, the zone present near the origin of the plasma pattern but not evident in the serum pattern may be regarded as being derived from fibrinogen. In interpreting this pattern it is difficult to decide with certainty whether the position of the fibrinogen zone is due to its having been converted to fibrin at the point of application (origin), as suggested by the circular configuration and size which has about the same dimensions as the circle resulting from the applied drop of plasma (about 0.01 ml.) at the beginning of the experiment, or is simply an expression of its low rate of diffusion and inherent electrical mobility, since in this pattern the point of origin falls coincidentally at a point intermediate between the gamma and beta globulins. It is of course well known that the fibrinogen boundary falls between these same constituents in conventional electrophoretic patterns obtained with sodium diethylbarbiturate buffer (Longsworth (9)).

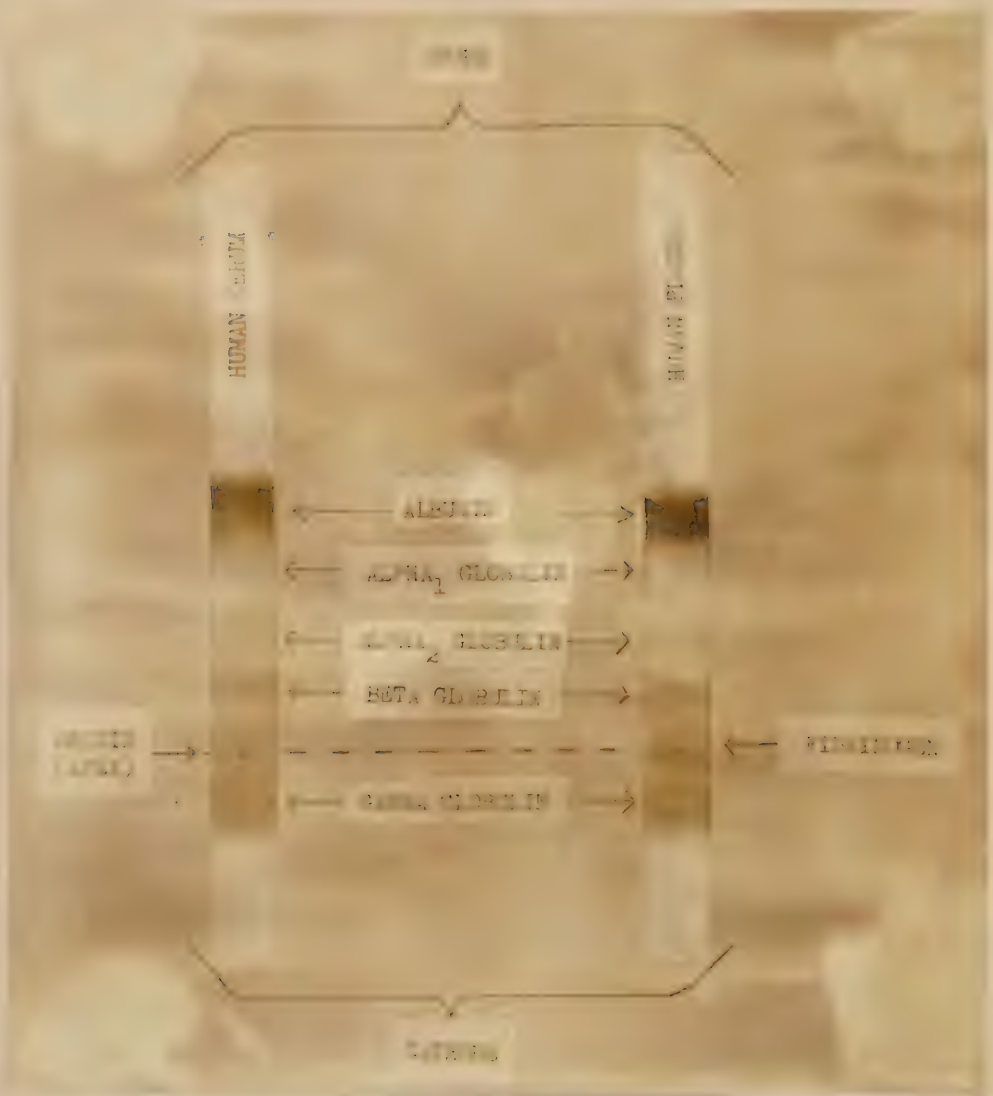
Experiment XI - Separation of radioactive inorganic iodide from protein bound iodine. A 230 gm. Wistar strain rat was injected intraperitoneally with 87 microcuries of I^{131}. The animal was sacrificed 210 minutes later. The thyroid was removed and all possible connective tissue carefully dissected from it. The resulting thyroid was ground in a Ten Broeck tissue grinder together with about 10 drops of 0.9% sodium chloride solution. The resulting material was centrifuged and the clear supernatant fluid applied to the reference marks of strips of filter paper which were separated in 0.05M. barbiturate buffer, pH 8.6, for various periods of time as illustrated in Figure 11. The resulting strips were dried in an oven for 5 minutes and then autoradiographs were made of the strips with varying exposures indicated.* The following points may be noted. In the experiments, a distinct band of radioactivity is visible migrating rapidly toward the anode. This undoubtedly corresponds to inorganic iodide ion and it is seen that, in a comparatively short time, the paper in the zones retaining protein (identified by its property of being coagulated and dyed) is completely "cleared" of inorganic iodide, the residual activity being associated with protein and/or amino acid fractions.

* The lower photograph is included to show detail in the protein-amino acid zones which is obscured by the longer exposure necessary to show the migration of the iodide ion.



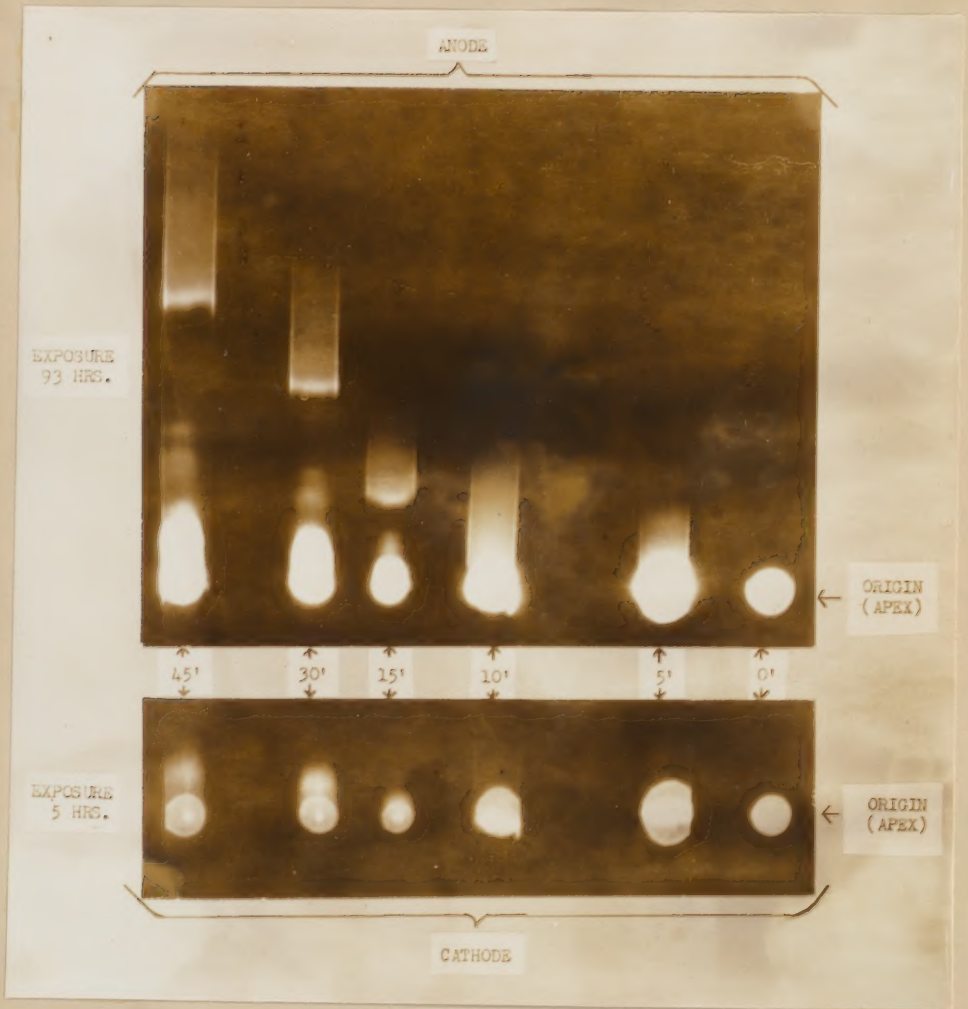
Electrolyte:	Phosphate Buffer (pH 7.6) 0.02M. (Sodium Phosphate Dibasic) (Sodium Phosphate Monobasic)	Barbiturate Buffer (pH 8.6) 0.05M. Sodium Diethylbarbiturat 0.01M. Diethylbarbituric Acid
Duration:	120 Minutes	180 Minutes
Current:	0.5 ma./cm. (width) Initial 400 Volts Final 340 Volts	0.5 ma./cm. (width) Initial 310 Volts Final 220 Volts
Paper:	Whatman No. 2 (2 Strips in Parallel in Apparatus of Fig. 1)	Whatman No. 2 (2 Strips of 5 run in Parallel Apparatus of Experiment III)
Apex Height:	14.5 cm.	11.5 cm.

FIG. 9 SEPARATION OF "IMMUNE GLOBULIN" (EXPERIMENT IX)



Electrolyte:	Barbiturate Buffer (pH 8.6) 0.05M. Sodium Diethylbarbiturate 0.01M. Diethylbarbituric Acid
Duration:	180 Minutes
Current:	0.5 ma./cm. (width)
Potential:	Initial 320 Volts Final 220 Volts
Paper:	Whatman No. 2 (2-1 cm. Strips in Series in 2 Cells)
Apex Height:	13.5 cm.

FIG. 10 COMPARISON OF HUMAN PLASMA AND SERUM PATTERNS (EXPERIMENT X)



Electrolyte:	Barbiturate Buffer (pH 8.6) 0.05M. Sodium Diethylbarbiturate 0.01M. Diethylbarbituric Acid
Duration:	As indicated above
Current:	0.5 ma./cm. (width)
Potential:	310 Volts (average)
Paper:	Whatman No. 2
Apex Height:	13.5 cm.

FIG. 11 AUTORADIOGRAPHS OF THYROID SEPARATIONS (EXPERIMENT XI)

III. DISCUSSION

In the technique employed, it is believed that the paper strip plays merely a passive role as a carrier of the electrolyte. It may be regarded as analogous in a limited sense to the Tiselius cell.

In the course of several hundred experiments, no evidence of adsorptive phenomena has been noted under the experimental conditions employed, in either the case of protein or amino acid separations. That is to say, (allowing for certain factors discussed below) the components seem to behave as they would be expected to in "free solution". Critical studies designed to answer this question have not been carried out and, therefore, the possible role of adsorption in the process must await elucidation. For this reason, it seems best for the time being to regard the process as one of ionophoresis or electrophoresis rather than "electrochromatography" as is the process described by Strain (1), or as "partition chromatography with applied voltage" as is the process described by Haugaard and Kroner (5).

It will be realized that under the experimental conditions employed a relatively complicated equilibrium obtains which include a number of simultaneously occurring processes which include at least the following:

a. Migration of ions due to the electrical field.
b. Diffusion.
c. Electroendosmotic flow.
d. Evaporation of water from the paper strip due to heating of the strip incidental to the current flow.
e. Hydrodynamic equilibrium on the paper strip between capillary forces and gravitational forces.
f. Electrical resistance changes along the length of the paper strip principally due to concentration effects secondary to factors d and e.

Sufficient data for a critical evaluation of these factors are not available. However, some of the more obvious relationships which appear to explain some of the experimental findings will be discussed briefly. We may consider that the field strength equation which is applied to the Tiselius cell is applicable as a first approximation at least to thin cross sections of the paper at any given level above the electrolyte level at any given instant. Then, migration velocity is proportional to field strength X and

$$X = \frac{I}{q\, k_s}$$

where I = current; q = cross sectional area of the paper; and k_s = conductivity of the solution on the paper at the cross section under consideration.

Limiting consideration to the case where the current is held constant, the cross section of the paper is constant and, therefore, must at any given level after equilibrium is established "contain" a given quantity of electrolyte. But, since the amount of electrolyte contained along the length of the paper varies due to the hydrodynamic and distillation equilibria mentioned,

the "effective cross section" of the paper may be regarded as increasing as the electrolyte level is approached, and as decreasing as the apex is approached, reaching its minimum "effective cross section" at the apex. Therefore, the field strength may be expected to be highest at the apex and to decrease as the electrolyte level in the electrode vessels is approached. The "drier" apex may be expected, therefore, to have more electrical resistance and, for a given current, would be expected to produce more heat than the "wetter" areas below. This factor would be expected to accentuate (or perhaps be principally responsible for) the "wetness gradient" down the paper. The above considerations appear to explain the lack of linearity of migration of ions with time as demonstrated for phenylalanine in Experiment VII (Figure 7).

It is for the above reasons that the apex height has been recorded in experimental data, it having been observed that reproducibility of the method could not always be achieved unless this factor were carefully controlled, especially with protein separations where a certain optimal "degree of wetness" of the paper for a given current and buffer seems to be essential for satisfactory resolution.

Under the experimental conditions employed, due to the very large surface area-electrolyte volume ration present in the paper strip, pronounced electroendosmotic currents toward the cathode would be anticipated. It is believed that this explains the apparent migration of the gamma globulin toward the cathode as illustrated in the serum and plasma patterns (Figures 8, 9, and 10. In conventional electrophoretic separations at pH 8.6, all the serum components are known to migrate to the anode. It is believed that this apparent migration of the gamma globulin toward the cathode can be explained by a displacement of the entire pattern toward the cathode due to this pronounced electroendosmotic current.

In view of the above considerations, a close correlation of the mobilities of protein constituents as measured in the Tiselius apparatus with those of these paper patterns is not to be expected.

It will be noted that the barbiturate buffer employed in these experiments is 0.05M. as compared with the 0.1M. buffer usually employed in conventional electrophoretic studies of human sera. It has been observed empirically that the more dilute buffer very much improves the degree of resolution attainable in human serum and plasma samples in the present technique. This may be due to the fact that the concentration of the buffer on the paper is increased above its value in the electrode vessels due to evaporation from the paper and, on the paper, thus approaches a concentration comparable with the optimum concentration observed in Tiselius separations.

VI. BIBLIOGRAPHY

1. Strain, H. H. On the combination of electrophoretic and chromatographic adsorption methods. J. Am. Chem. Soc. 61, 1292, 1939.

2. Coolidge, T. B. A simple cataphoresis apparatus. J. Biol. Chem., 127, 551, 1939.

3. Consden, R., A. H. Gordon and A. J. P. Martin. Ionophoresis in silica jelly. Biochem. J. 40, 33, 1946.

4. Butler, J. A. V. and J. M. L. Stephen. An apparatus for preparative electrophoresis. Nature 160, 469, 1947.

5. Haugaard, G. and T. D. Kroner. Partition chromatography of amino acids with applied voltage. J. Am. Chem. Soc. 70, 2135, 1948.

6. Consden, R., A. H. Gordon and A. J. P. Martin. Qualitative analysis of proteins: A partition chromatographic method using paper. Biochem. J. 38, 224, 1944.

7. Williams, R. J. and H. Kirby. Paper chromatography using capillary ascent. Science 107, 481, 1948.

8. Moore, D. H. and J. U. White. A new compact Tiselius electrophoresis apparatus. Rev. Sci. Inst. 19, 700, 1948.

9. Longsworth, L. G. Recent advances in the study of proteins by electrophoresis. Chem. Rev. 30, 323, 1942.